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PRINCIPAL INVESTIGATOR: Ronald J. Weigel, M.D., Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-4125

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An important transcriptional regulatory element of the estrogen receptor (ER) gene has been identified that binds a protein expressed in ER-positive breast carcinoma. Using a transient expression assay, a seventy-five base pair region of the 5' untranslated leader of the ER gene was identified which augments expression from the ER promoter. This region contains two binding sites for a protein, estrogen receptor factor-1 (ERF-1), which is expressed in ER-positive breast carcinomas. A concatenated ERF-1 binding site probe identified a 30,000 dalton protein. Low level ERF-1 expression was detected in normal human mammary epithelial cells. Abundant ERF-1 expression was also found in endometrial carcinoma cell lines that express the ER-positive phenotype. These results indicate that ERF-1 expression represents a common mechanism of ER regulation in hormonally responsive carcinomas.

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Ronald Wenger   10.13.95

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INTRODUCTION

Background

The expression of estrogen receptor (ER) is intimately associated with the biology of breast carcinoma. Breast carcinomas occurring in postmenopausal women are often ER-positive (8) and many of these tumors express significantly more receptor than normal mammary epithelium (17). ER-negative breast carcinomas are more likely to occur in young women and these tumors carry a worse prognosis than carcinomas which express ER (12,3). Several studies have focused on the function of ER in an attempt to explain the association between ER expression and tumor biology. Mutations have been described in the ER gene of some breast carcinomas that render these altered ER proteins incapable of binding estrogen response elements (ERE)(21,19) and able to inhibit wild-type ER function (9,5). Other studies, however, have found ER mutations which result in a constitutively active receptor which has also been postulated as important to the development of hormone-independent growth (14,2). If ER function is influencing the oncogenic process, it is difficult to conceptualize within a single model of oncogenesis the occurrence of mutations which inhibit ER function and mutations which result in constitutive activity. An alternative hypothesis is that mechanisms regulating transcription of the ER gene influence the phenotype of breast carcinoma; within this model, ER-negative carcinomas which do not transcribe the ER gene define a subset of tumors with a more aggressive phenotype. This theory is supported by recent studies which have identified breast carcinoma cell lines that fail to transcribe an apparently normal ER gene (24). It is therefore possible that defining molecular mechanisms controlling transcription of the ER gene may provide new insight into the biology of breast carcinoma.

Transcription of ER occurs from two separate promoters, P0 and P1 (10), although no functional mapping has been previously published. P1 is the major ER transcriptional start site (7). The P1 cap site is predominantly utilized in human mammary epithelial cells (HMEC) and is the major start site in ER-positive human breast carcinomas (23). Multiple cap sites have been identified for the P0 promoter. Studies of the murine ER gene identified 10 cap sites spanning approximately 60 bases (25) and a start site at -1994 (from the P1 cap site) was identified in human cells which would agree closely with the major murine P0 cap site (16). There is possibly another ER transcriptional start site farther upstream at -3090 (16). Transcription from the P0 promoter is characteristic of human endometrial tissue and can account for 12 to 33% of ER transcription in breast carcinoma cells (23).

Purpose of Present Work

These experiments are designed to investigate mechanisms regulating transcription of the ER gene. The experiments described address the technical objectives 1 and 2 of this U.S. Army Research Grant DAMD17-94-J-4353. Several breast carcinoma cell lines, normal breast epithelial cells and endometrial carcinoma cell lines are analyzed in an attempt to correlate transcription factor expression with ER expression. Mutational analysis of the ER promoter is also performed to correlate binding of cellular factors with transcriptional regulation.

Methods of Approach

Cell Lines. Human mammary epithelial cells (HMEC) were obtained from reduction mammoplasties and maintained in DFCI-1 growth media as described (1). The ECC-1 ER-positive human endometrial carcinoma cell line was obtained from Dr. Satyaswaroop, Hershey, PA. All other cell lines were obtained from American Type Culture Collection, Rockville, MD.

Cells were maintained in minimal essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine sera (Hyclone, Logan, UT), 25mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid, 26mM sodium bicarbonate, 5000 units/ml penicillin G (Gibco BRL), 5000μg/ml streptomycin (Gibco BRL), and 6ng/ml bovine insulin (Sigma Chemical Company, St. Louis, MO). Cells were incubated at 37°C in 5% CO_2.

Plasmid Construction and Transfection. The region of DNA in the 5' end of the ER gene was isolated from a human genomic lambda library (Stratagene, La Jolla, CA). The 5' end was sequenced and a set of primers were constructed, all of which contained a *Bgl* II site for use as upstream primers. All 3' oligonucleotides contained a *Hin*d III site. These oligonucleotide primers were used in polymerase chain reaction with cloned DNA as template. Primers used to generate 5' deletion constructs were as follows:

ER724: 5' -TACAGATCTG TGGTCCAACA TAAACACA

ER586: 5' -TGCAGATCTT CCTATATGTA TACCC

ER464: 5' -CATTAGATCT GCCCTATCTC GGTTACAGTG T

ER375: 5' -GGGGAGATCT AACAGAAAGA GAGACAA

ER289: 5' -CCCTAGATCT GTCTTTCGCG TTTAT

ER122: 5' -GGGAGATCTG CCTGGAGTGA TGTTTAAG

ER40: 5' -TATGAGATCT GGAGACCAGT ACTTAAAG

ER0: 5' -CCCAGATCTG GCGGAGGGCG TTCG

Primers used to generate specific 3' ends were:

+230: 5' -CATAAGCTTG GTCCGTGGCC GCGGGCAGGG T

+210: 5' -CGGGAAGCTT GCAGACCGTG TCCCCGCAGG

+135: 5' -GCCCAAGCTT AGAGGCGACG CAGCGCA

+0: 5' -CGCCAAGCTT CCTGGGCTCC CGGGCCTC

PCR products were then subcloned into the *Bgl* II-*Hin*d III sites of the luciferase reporter plasmid pGL2-Basic (Promega, Madison, WI). To construct the plasmids with 3500 bp upstream of P1, the 5' region was first subcloned into pBluescript from the genomic lambda clone as a *Sal*I - *Not*I fragment utilizing the *Sal*I site in lambda DASH and the *Not*I site in the first exon of ER. The ER724-xLUC constructs were each digested with *Xho*I - *Nde*I removing the 5' region of the ER gene. This region was then replaced with the larger 5' flanking region as an *Xho*I - *Nde*I fragment from the ER gene cloned in pBluescript. In this construct the *Xho*I site 6 bp upstream of the *Sal*I site in pBluescript was utilized and was inserted into the *Xho*I site 4 bp upstream of the *Bgl*II site in pGL2-Basic. Each of these plasmids then contain 3500 bp upstream of P1.

Plasmid DNA was prepared using alkaline lysis and purified using polyethylene glycol as described (18). All cells were transfected using the calcium phosphate precipitation procedure (6). In these experiments 30μg of cloned plasmid DNA was used in transfections of 100mm plates with cells at 50-60% confluence. In each transfection, 2μg of an RSV-driven CAT expression vector was co-transfected and cells were assayed for luciferase and CAT expression 48 hours after transfection. Values presented are luciferase units as measured on a luminometer corrected for transfection efficiency, as determined by CAT assay. Similar results were obtained when 15μg of plasmid DNA was used in transfections.

Gel Retardation Assay. Cells were collected by dislodging with a policeman or trypsinization. Cell pellets were washed with 1 x PBS and then resuspended at 10^8 cells/ml in

microextraction buffer [450 mM NaCl, 20 mM HEPES, pH 7.7, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and proteinase inhibitors]. Cells were sonicated and cellular debris was pelleted at 14,000 rpm in a microfuge. Protein concentration of the supernatant was determined using a Bio-Rad protein assay (Hercules, CA) and ran in the range of 5-15 mg/ml. Extracts were stored at -80°C until use.

Ten micrograms of whole cell extract was incubated in 1x binding buffer [40 mM KCl, 20 mM HEPES, pH 7.7, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM DTT], 4% Ficoll, 40μg/ml poly dI-dC, 0.1 ng of radiolabelled probe in a volume of 25 μl at room temperature for 45 minutes. Reactions were then loaded on a 4% acrylamide gel in 0.25x TBE and electrophoresed at 270 volts at 4°C. Gels were dried and exposed to x-ray film.

Double stranded DNA oligonucleotides were used for gel-shift competition and were prepared by separately synthesizing each strand of the oligonucleotide using a Gene Assembler Special (Pharmacia LKB, Alameda, CA). Oligonucleotides were mixed in equal molar ratio in 0.3M sodium acetate, boiled for 10 minutes and cooled slowly to room temperature. Double stranded oligonucleotide DNA was then ethanol precipitated and resuspended in water at a concentration of 0.2mg/ml. These double-stranded oligonucleotides were then used in gel shift competitions at 500M excess.

Southwestern Blot. Approximately 70μg of total cell protein was electrophoresed in 12% SDS-PAGE, transferred to nitrocellulose and protein was renatured using guanidine hydrochloride as described (18). Probes were labelled with α^{32}P-dCTP using the random primer kit (Boehringer-Mannheim, Indianapolis, IN).

RESULTS

Functional Analysis of the ER Promoter

To elucidate the mechanisms which regulate transcription of the ER gene, we have performed a functional analysis of the ER promoter. A human genomic lambda library was screened with a probe from the 5' flanking region of the ER gene. A genomic clone was obtained which contained 3500 bp upstream of the P1 cap site for ER, the entire first coding exon, and approximately 10 kbp of the first intron. This 5' flanking region encompasses 500 bp upstream of the farthest ER cap site identified. Regions of this clone were then subcloned into the luciferase reporter vector pGL2-Basic. Figure 1 shows a diagram of the promoter constructs. The major ER mRNA beginning at P1 contains a 230 base untranslated 5' leader sequence (7). In the first set of constructs, all inserts contained 210 bp of the untranslated leader and a nested set of 5' deletions were generated from 3500 bp down to the P1 cap site at +1 (Figure 1A.) In a second set of promoter constructs, the 5' end remained at 3500 bp and 3' deletions were constructed beginning at +230 and progressively deleting portions of the leader by sequentially bringing the luciferase gene closer to the P1 cap site (Figure 1B.)

These constructs were tested for luciferase expression upon transfection into two breast carcinoma cell lines (Figure 2). T47D is an ER-positive breast carcinoma line in which approximately 90% of the ER mRNA begins at the P1 cap site (23). The ER-negative carcinoma line, MDA-MB-231 was also used since we have previously shown this cell line to lack transcription of the ER gene (24). Luciferase activity in these two cell lines demonstrated strikingly different results (Figure 2). In ER-positive T47D, the full-length construct gave excellent expression. Progressive deletion of the 5' end of the gene failed to significantly alter

expression, although there was a reproducible decline of expression upon deletion of the last 40 bp of the 5' flanking region which contains a TATA element at +25 (Figure 2A.) The pattern of expression in ER-negative MDA-MB-231 was qualitatively and quantitatively different. The full-length construct expressed poorly and gave values an order of magnitude less than in T47D. This level of expression was only three times the expression from the negative control vector pGL2-Basic, which does not contain a promoter. Progressive deletion of the upstream sequences improved expression and no significant decline occurred when the TATA element upstream of the P1 cap site was deleted. Results from the 3' deletion constructs are shown in Figure 2B. Deletions from the 3' end of the leader identified a sequence between +210 and +135 which augmented expression of the ER promoter in ER-positive T47D cells. Analysis of these constructs in ER-negative MDA-MB-231 was also performed and the effect of this region appeared to be cell line specific. We, therefore, searched for binding proteins that interact with this region of the ER gene.

Identification of ERF-1 by Gel Shift

An 80 bp probe was prepared from sequences from +132 to +211 of the ER promoter. This probe was used in gel-shift assays with extracts prepared from the ER-positive breast carcinoma cell lines MCF7 and T47D and the ER-negative MDA-MB-231 cell line. A prominent shift band was found only in extracts from the ER-positive cell lines (Figure 3). This complex is referred to as ERF-1 (Estrogen Receptor Factor-1). Gel shift competitions were used to identify two ERF-1 binding sites within this 80 bp region. Cold competitor prepared from the entire 80 bp probe efficiently competes for binding (Figure 3). The region from +132 to +171 partially competes and +172 to +211 competes efficiently. Within the region from +172

to +211, the sequences from +182 to +201 compete efficiently for binding (Figure 3). Neither +172 to +191 nor +192 to +211 demonstrate any competition. These results indicate that a DNA binding protein, ERF-1, is expressed in ER-positive breast carcinoma cells and interacts with a region of the leader with transcriptional activity. ERF-1 binds to two sites in this region, a distal (high affinity) and a proximal (low affinity) site.

ERF-1 Binding Sites

The distal binding site was mapped precisely utilizing gel shift competition with oligonucleotides containing mutations within the sequences from +182 to +201. These results are shown in Figure 4A. This region contains the sequence CCCTGCGGGG which is an imperfect palindrome. The wild-type sequence of this distal site (dwt) competes efficiently. Mutations d1 and d3 disrupt the imperfect palindrome and destroy the ability of the oligonucleotide to compete. Mutations d2 and d4 do not alter the 10 bp imperfect palindrome and retain the ability to compete for binding although d4 is slightly less efficient than dwt. Mutation d5 changes the T at +192 to a C and converts the sequence to a perfect palindrome but partially weakens the ability to efficiently compete. The sequence between +132 to +171 that demonstrated weak competition shown in Figure 3, was found to contain a second ERF-1 site located at +130 to +149 (Figure 4B). Within this region, a related sequence can be found and homologous mutations as used for the distal site have identical consequences for binding. Interestingly, insertion of a G between +140 and +141 creates a site identical to the distal site. This mutation improves the ability of the weak proximal site to compete.

Mutation of ERF-1 Sites Affects Expression

Because the mutation p1 destroys binding to the proximal site and d1 destroys binding

to the distal site, these two mutations were built into the expression vector ER3500-230LUC and called ER3500-230p1d1. This new vector is identical to ER3500-230LUC except for the two mutations within the proximal and distal ERF-1 binding sites. Expression from this construct is shown in Figure 2B. Mutation of these ERF-1 sites has an effect on expression similar to deletion of the region from +135 to +210. These results strongly suggest that ERF-1 is a transcription factor which is expressed in ER-positive breast carcinoma and which functions by binding to two sites in the untranslated leader of the ER gene.

Identification of ERF-1 by Southwestern Blot

A complex formed in a gel shift assay often contains a number of proteins. To define the ERF-1 specific binding protein, protein blots of crude cell extracts were prepared from MCF7 and MDA-MB-231 cells. A blot was reacted with a radiolabelled DNA probe from sequences +100 to +230 of the ER leader. This probe identified a 35 kD protein present in both MCF7 and MDA-MB-231 and a second protein of approximately 30 kD only found in MCF7 (Figure 5). An identical blot was reacted with a probe prepared from a concatenated high-affinity distal ERF-1 binding site. This probe identified only the p30 protein found in MCF7 cells. These results indicate that the specific ERF-1 binding protein is a 30 kD protein expressed in MCF7 but not MDA-MB-231. Alternatively, the protein may be expressed in MDA-MB-231 but is modified in such a way as to prevent DNA binding.

ERF-1 Expression in Breast and Endometrial Carcinoma

To determine which cell lines express ERF-1, a panel of human cell lines were analyzed for ERF-1 expression using the gel shift assay. These results are shown in Figures 6A and B. Abundant ERF-1 expression was found in all ER-positive breast carcinoma cell lines tested -

MCF7, T47D, and BT20. Low levels of ERF-1 were detected in normal human mammary epithelial cells (HMEC). ERF-1 complex from HMEC demonstrated identical binding sequence specificity as the complex in MCF7 and T47D (data not shown). HBL-100 is an ER-negative breast carcinoma line which appears to express low amounts of ERF-1 comparable to HMEC. The lack of expression of ER in HBL-100 could be due to any one of a number of reasons, e.g. methylation of DNA, deletion of the ER gene, rapid degradation of mRNA.

Expression of ERF-1 was also examined in other cell lines, including a panel of human endometrial carcinoma lines. The RL95-2 cell line is an endometrial adenocarcinoma line which is reportedly ER-positive (22). This cell line makes abundant ERF-1 protein as seen in Figure 6B. Examination of ER expression in this stock of RL95-2 has failed to demonstrated ER expression (data not shown); however, late passage of this cell line has been reported to lose ER expression (20). Therefore, RL95-2 was derived from an ER-positive carcinoma and loss of ER expression as the cells are maintained in culture is likely the result of DNA methylation. ECC-1 is another endometrial cell line which is ER-positive and we have confirmed expression of ER mRNA (data not shown). The ECC-1 cell line also expresses abundant ERF-1 (Figure 6B). ERF-1 was not readily detected in HEC 1B or HEC 1A, which are both ER-negative human endometrial carcinoma cell lines (13,11). These results suggest that abundant expression of ERF-1 represents a common mechanism for ER regulation in hormonally responsive carcinomas.

Discussion

Although several mechanisms are involved in controlling expression of ER in breast carcinomas, transcriptional regulation is clearly responsible for the ER-negative phenotype in

some cell lines (15,24). These data demonstrate that an important transcriptional regulatory element of the ER gene is found within the 5' untranslated leader. This region contains two binding sites for a DNA binding protein, ERF-1, which is abundantly expressed in ER-positive breast and endometrial carcinomas. These data indicate a role for ERF-1 in the transcriptional regulation of ER. There are likely other cis-acting control elements involved in the regulation of ER transcription. For example, the transcriptional mapping data also implicate positive and negative regulatory elements upstream of the P1 transcriptional start site and there may be other regions that have not been examined in the present study. However, the identification of ERF-1 offers a molecular mechanism that accounts for differences in ER expression found in breast carcinomas.

The complexity of ER transcriptional regulation is suggested by the biology of ER expression. ER is over-expressed in many ER-positive breast carcinomas that often make over 100 fmol/mg cytosol protein compared to 4 fmol/mg cytosol protein in normal mammary cells (17). These differences in ER expression are reflective of mRNA levels and there are striking differences in the level of ER mRNA detected in different breast carcinoma lines (23). It is certainly possible that ERF-1 may be responsible for ER over-expression identified in many ER-positive carcinomas. However, more detailed experiments need to be performed to address this possibility directly.

Comparing the sequence of the ERF-1 binding site to known transcription factor sites fails to identify a previously identified factor (4). There can be a high degree of degeneracy in binding site sequences, but none of the known factors with GC rich sites has a molecular weight of 30 kD. Since ERF-1 expression is limited to ER-positive cell lines, one might wonder

whether ER is part of the ERF-1 complex. Several facts make this unlikely. First, the ERF-1 site bears no resemblance to the estrogen response element (ERE). Secondly, BT20 expresses a truncated ER with a molecular weight of 43 kD (2) but generates a ERF-1 complex which co-migrates with other ER-positive cell lines. Thirdly, the Southwestern blot in Figure 4 identifies a protein of 30 kD which is clearly distinct from the 65 kD ER protein. Finally, attempts to super-shift the ERF-1 complex with an antibody against ER have not been successful (data not shown).

CONCLUSION

The identification of ERF-1 offers new insight into our understanding of the relationship between ER expression and the biology of breast and endometrial carcinoma. ER expression defines a subset of breast cancer patients who, in general, have a better prognosis compared to patients with ER-negative tumors. Because ER is a transcription factor, it has been suggested that the phenotype displayed by ER-positive breast carcinomas is due to the repertoire of genes whose expression is regulated through estrogen response elements (ERE). Alternatively, ER expression might be a marker for the degree of differentiation of a tumor and ERF-1 might be involved in the regulation of a number of cellular genes, including ER, which are critical to the differentiated phenotype. The identification of ERF-1 has immediate clinical relevance. For example, tumors that lack ERF-1 expression might define a subset of cancer patients with a prognosis different than patients with ER-negative tumors where loss of expression is due to mutations within the ER gene. If ERF-1 expression is a more reliable marker of a clinically relevant phenotype, this would indicate that some ERF-1 responsive gene, other than ER, is critical to the phenotype of ER-positive carcinomas. Understanding the control of ERF-1 may

also provide new therapeutic approaches to the treatment of aggressive ER-negative tumors.

The next objective of this research project is to clone the ERF-1 gene. We are currently developing a protein purification strategy to purify ERF-1 protein from MCF7 cells. This protein will be sequenced and the gene cloned as described in our grant proposal (objective 3). Once this is accomplished, ERF-1 will be examined for its role in ER transcriptional regulation.

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APPENDIX

Figure Legends

Figure 1. ER promoter constructs.

Diagram of ER promoter constructs cloned in luciferase expression vector pGL2-Basic. Dark line represents DNA sequence derived from ER gene. Arrow shows location of P1 transcriptional start site. All numbers correspond to distance from P1 cap site. Location of *Nde*I site at -45 and *Xho*I site (in vector) are shown. ER coding region is indicated in box. Luciferase coding region is designated by box labelled LUC. In plasmid names, first number corresponds to DNA length upstream of P1 and second number is DNA length downstream of P1. A. 5' deletion constructs are shown. All these constructs contain 210 bp of the 230 bp 5' untranslated leader. B. 3' deletion constructs are shown. All the constructs contain 3500 bp upstream of P1 and variable portions of the untranslated leader.

Figure 1A

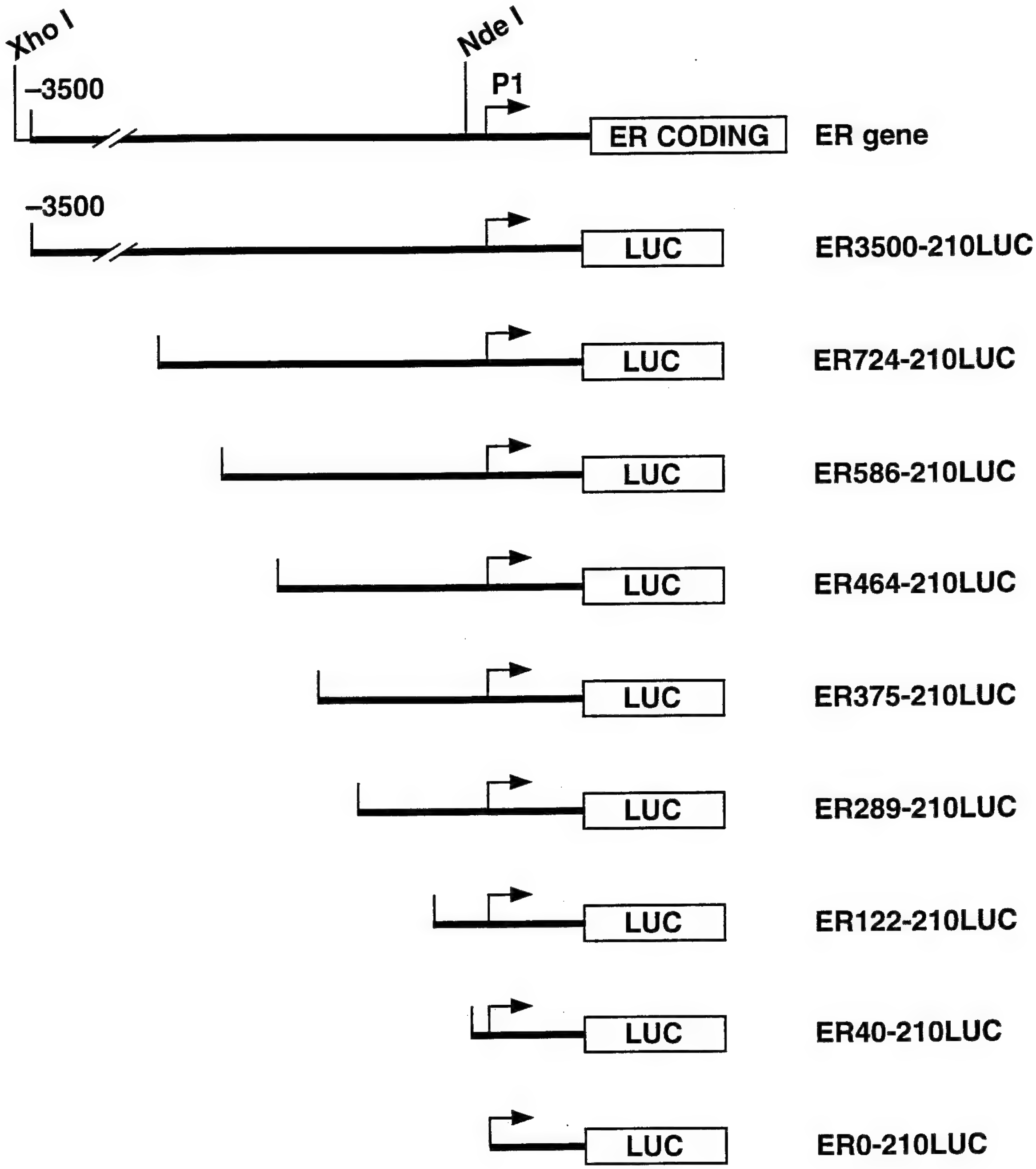
Xho I
Nde I
–3500
P1
ER CODING
ER gene
–3500
LUC
ER3500-210LUC
LUC
ER724-210LUC
LUC
ER586-210LUC
LUC
ER464-210LUC
LUC
ER375-210LUC
LUC
ER289-210LUC
LUC
ER122-210LUC
LUC
ER40-210LUC
LUC
ER0-210LUC

Figure 1B

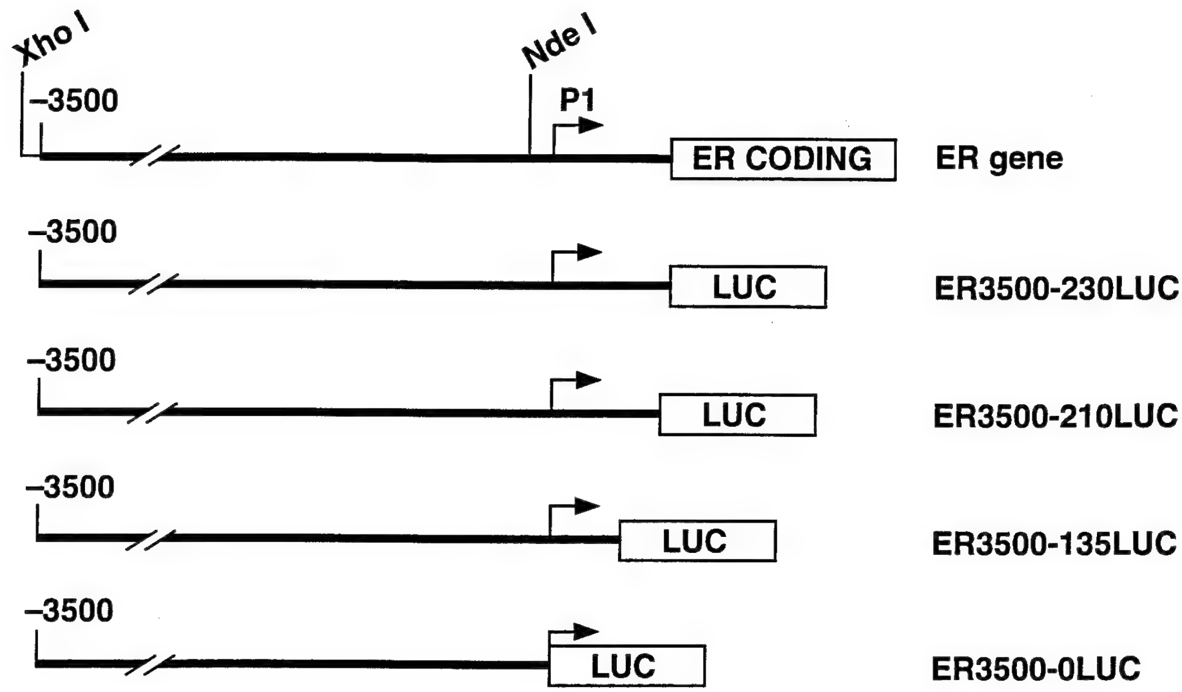
Xho I
Nde I
-3500
P1
ER CODING
ER gene
-3500
LUC
ER3500-230LUC
-3500
LUC
ER3500-210LUC
-3500
LUC
ER3500-135LUC
-3500
LUC
ER3500-0LUC

Figure 2. Luciferase activity from ER promoter constructs.

Plasmid DNA was transfected into T47D (ER-positive) or MDA-MB-231 (ER-negative) breast carcinoma cells and assayed for luciferase activity. Data presented is corrected for transfection efficiency. Data was normalized with the activity obtained from the largest plasmid in T47D as 100%. Standard error is shown by error bars. (A) Representative results of expression from 5' deletion constructs. Values shown are average of four separate transfection experiments. (B) Representative results of expression from 3' deletion constructs. Values shown are average of eight separate transfections. Data for ER3500-230p1d1 is average of two separate transfections performed in triplicate and is representative of other transfections performed.

Figure 2A

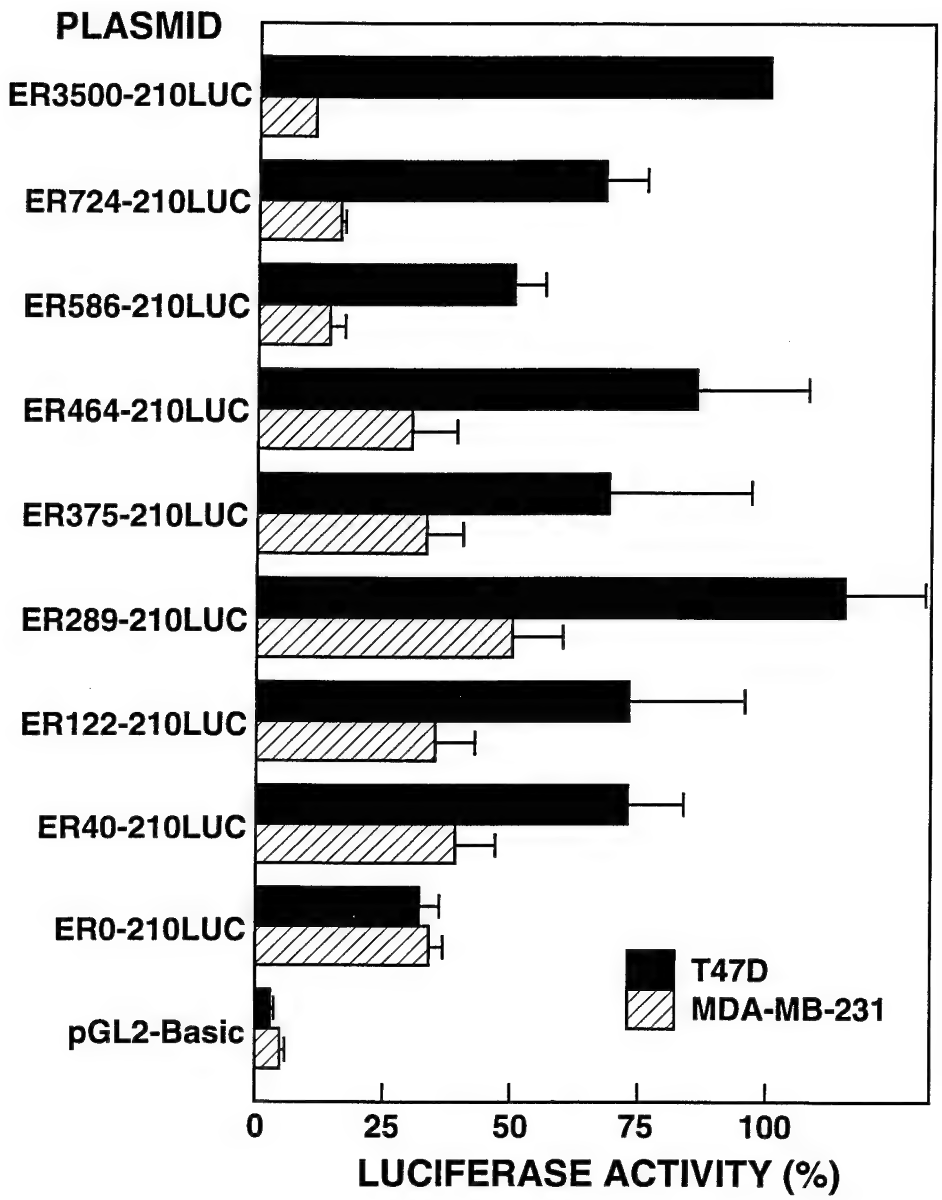
PLASMID
ER3500-210LUC
ER724-210LUC
ER586-210LUC
ER464-210LUC
ER375-210LUC
ER289-210LUC
ER122-210LUC
ER40-210LUC
ER0-210LUC
pGL2-Basic
T47D
MDA-MB-231
0
25
50
75
100
LUCIFERASE ACTIVITY (%)

Figure 2B

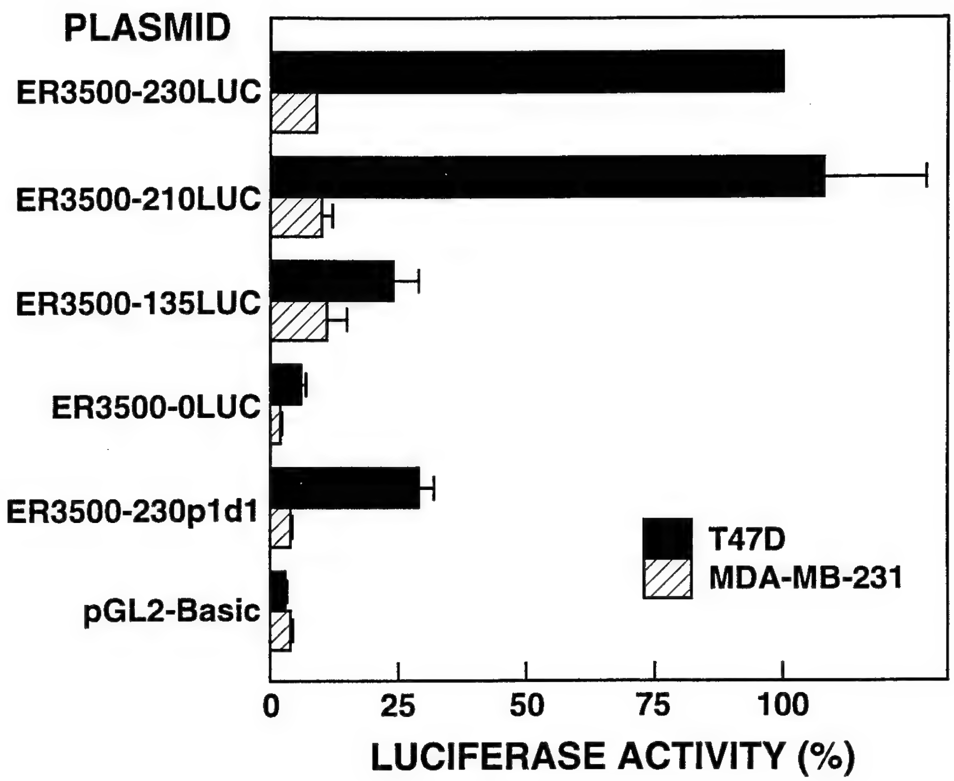
PLASMID
ER3500-230LUC
ER3500-210LUC
ER3500-135LUC
ER3500-0LUC
ER3500-230p1d1
pGL2-Basic
T47D
MDA-MB-231
0
25
50
75
100
LUCIFERASE ACTIVITY (%)

Figure 3. Gel shift assay using 80 bp probe.

Gel shift assay was performed using whole cell extracts from cells as shown. The probe is a radiolabelled 80 bp DNA fragment from ER promoter sequences +132 to +211. Gel shift competition involved addition of 500 fold molar excess cold competitor from sequences of ER gene as shown. In (-) lanes there is no competitor added.

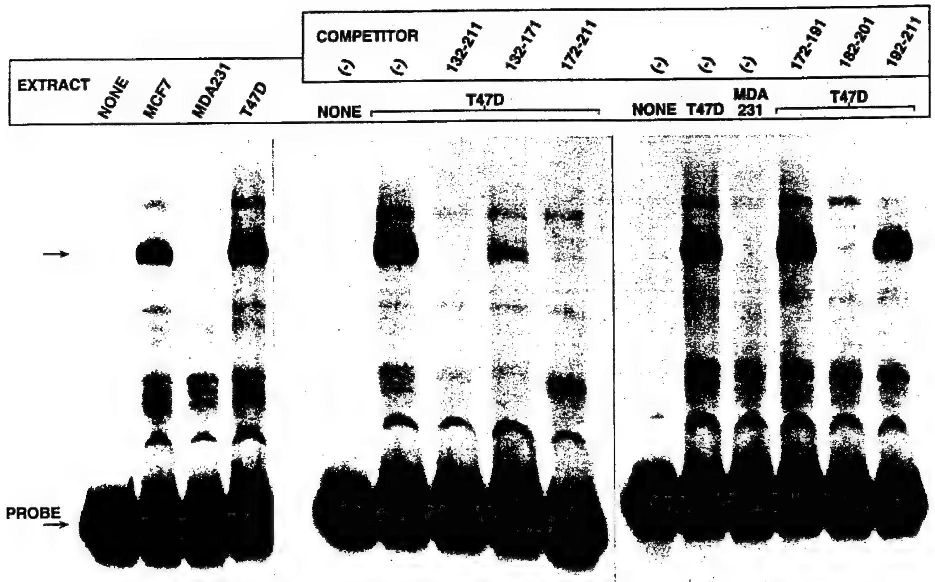
COMPETITOR
(-)
(-)
132-211
132-171
172-211
(-)
(-)
(-)
172-191
182-201
192-211
EXTRACT
NONE
MCF7
MDA231
T47D
NONE
T47D
NONE
T47D
MDA 231
T47D
PROBE

Figure 3

Figure 4. Mapping ERF-1 binding sites.

Gel shift competitions were performed using whole cell extract from cells shown. Probe used in both A and B is a 72 bp DNA fragment from ER promoter sequences +130 to +201. (A) Competitions using mutant distal binding sites. Sequence of wild-type distal site (dwt) and position of mutated sequence is shown for each mutant sequence d1 - d5. (B) Proximal binding site defined using proximal mutants p1 - p4. Below gel is shown sequences of distal site (dwt) and proximal site wild-type sequence aligned to highlight homology. Sequence of proximal site wild-type sequence (pwt) and four proximal site mutants p1 - p4 is shown.

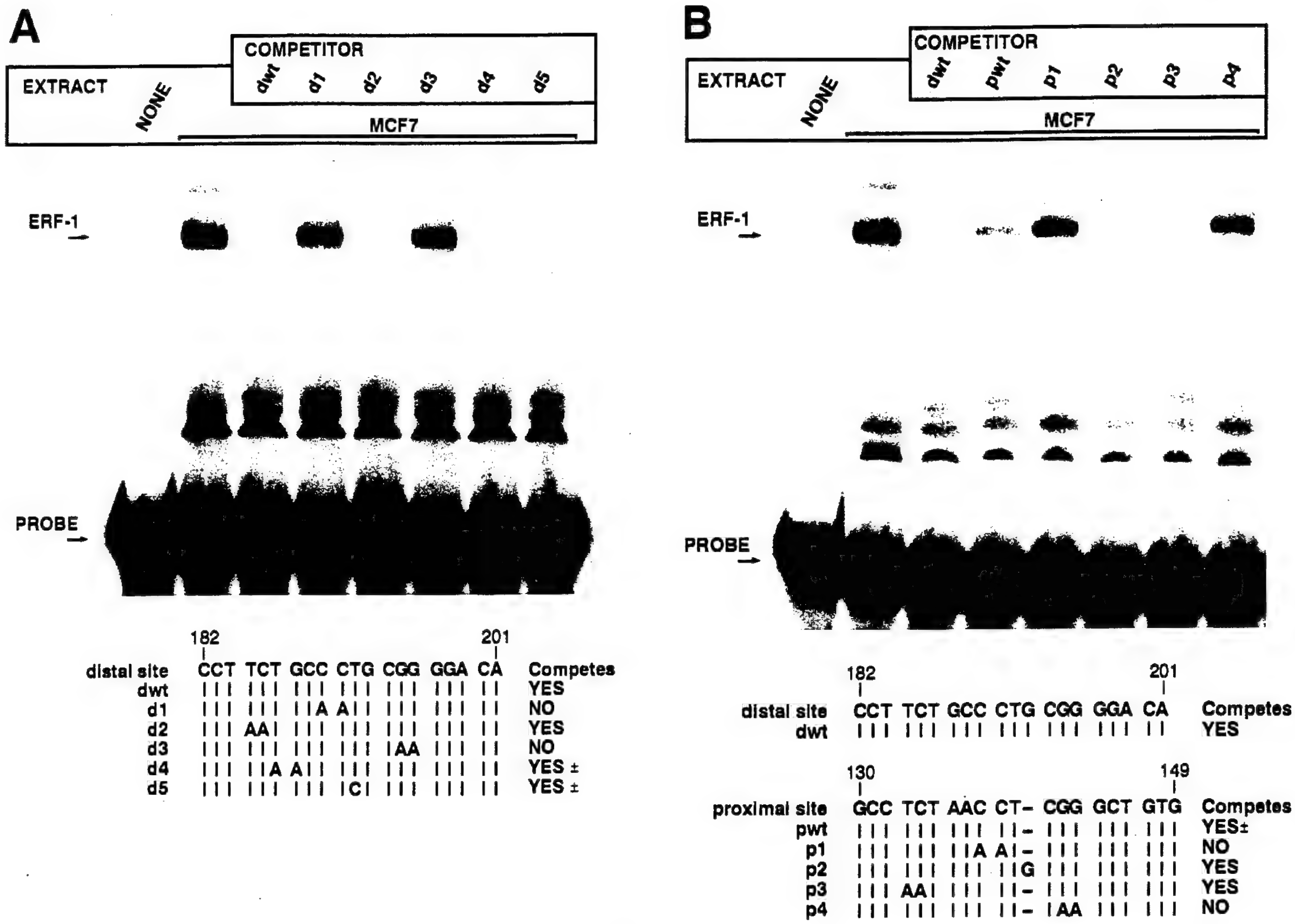
A
EXTRACT
NONE
COMPETITOR
dwt
d1
d2
d3
d4
d5
MCF7
ERF-1
PROBE
182
201
distal site CCT TCT GCC CTG CGG GGA CA Competes
dwt ||| ||| ||| ||| ||| ||| || YES
d1 ||| ||| ||A A|| ||| ||| || NO
d2 ||| AA| ||| ||| ||| ||| || YES
d3 ||| ||| ||| ||| |AA ||| || NO
d4 ||| ||A A|| ||| ||| ||| || YES ±
d5 ||| ||| ||| |C| ||| ||| || YES ±
B
EXTRACT
NONE
COMPETITOR
dwt
pwt
p1
p2
p3
p4
MCF7
ERF-1
PROBE
182
201
distal site CCT TCT GCC CTG CGG GGA CA Competes
dwt ||| ||| ||| ||| ||| ||| || YES
130
149
proximal site GCC TCT AAC CT- CGG GCT GTG Competes
pwt ||| ||| ||| ||- ||| ||| ||| YES±
p1 ||| ||| ||A A|- ||| ||| ||| NO
p2 ||| ||| ||| ||G ||| ||| ||| YES
p3 ||| AA| ||| ||- ||| ||| ||| YES
p4 ||| ||| ||| ||- |AA ||| ||| NO

Figure 4

Figure 5. Southwestern blot of protein extracts.

Cell extracts from MCF7 or MDA-MB-231 were used on PAGE as shown. On left, the protein blot was probed with a DNA fragment from +100 to +230 region of ER promoter. On right, an identical protein blot was probed with a concatenated distal ERF-1 binding site from sequences +182 to +201 of ER promoter.

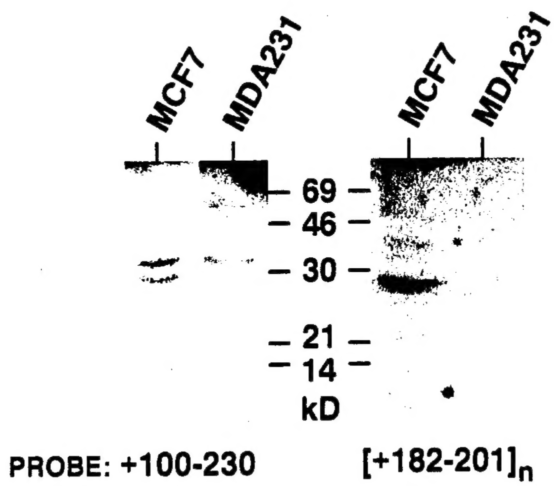
MCF7
MDA231
MCF7
MDA231
69
46
30
21
14
kD
PROBE: +100-230
[+182-201]n

Figure 5

Figure 6. ERF-1 expression in human cell lines.

ERF-1 expression determined by gel-shift assay using whole cell extracts prepared from cell lines shown and reacted with a 72 bp probe encompassing proximal and distal ERF-1 sites. (A) Human breast carcinoma lines; ER-positive: MCF7, T47D, BT20 and ER-negative: MDA-MB-231 and HBL-100. HMEC are normal human mammary epithelial cells. (B) Other human cell lines; HeLa are cervical carcinoma cells; Daudi are lymphocytes; RL95-2, HEC 1B, HEC 1A, and ECC-1 are human endometrial carcinoma cell lines (see text for details).

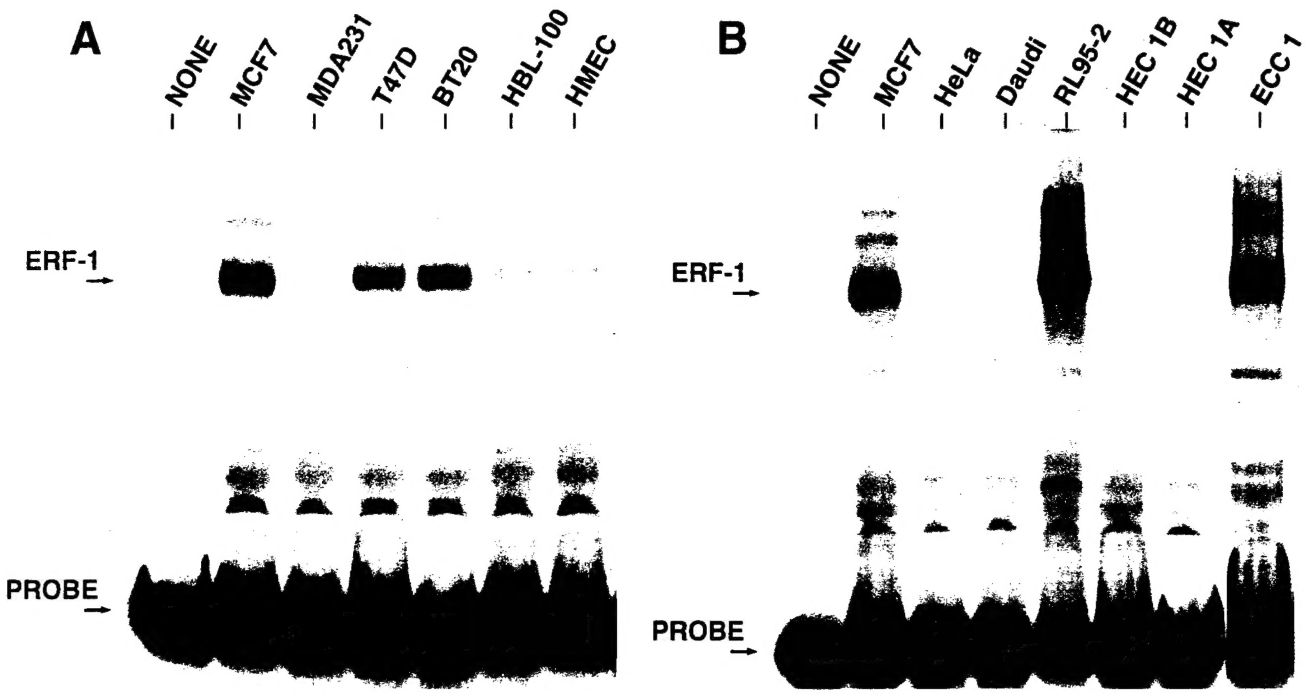
A
NONE
MCF7
MDA231
T47D
BT20
HBL-100
HMEC
ERF-1
PROBE
B
NONE
MCF7
HeLa
Daudi
RL95-2
HEC 1B
HEC 1A
ECC 1
ERF-1
PROBE

Figure 6